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Retrovirus DNA Termini Bound by Integrase Communicate *in Trans* for Full-Site Integration *in Vitro*

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Integration of linear retrovirus DNA involves the concerted insertion of the viral termini (full-site integration) into the host chromosome. We investigated the interactions that occur between long terminal repeat (LTR) termini bound by avian retrovirus integrase (IN) for full-site integration *in vitro*. Wild-type (wt) or mutant LTR donors that possess gain-of-function ("G") or loss-of-function ("L") for full-site integration activity were used. G LTR termini are characterized as having significantly higher strand transfer activity than the wt and the L LTR termini. L LTR mutations are classified as partially or extremely defective for strand transfer activity. The L mutations were further classified by their ability to either permit or block the assembly of G or wt LTR termini into nucleoprotein complexes capable of full-site strand transfer. We demonstrated that avian myeloblastosis virus IN bound to G LTR termini increased the incorporation of partially defective L LTR termini into nucleoprotein complexes that were capable of full-site integration. The observed full-site integration activity of these assembled nucleoprotein complexes appeared to be influenced by each individual IN–LTR complex *in trans*. In contrast, extremely defective L LTR termini exhibited the ability to effectively block the assembly of wt LTR termini into nucleoprotein complexes capable of full-site strand transfer. Data from nonspecific DNA competition experiments suggested that IN had an apparent higher affinity for G LTR donor termini than for partially defective L LTR donor termini as measured by full-site integration activity. However, assembled nucleoprotein complexes containing either two G or two L LTR donors were stable, having a similar half-life of ~2 h on ice. The results suggest that LTR termini bound by IN exhibit an allosteric effect to modulate full-site integration *in vitro*. Similar regulatory controls also appear to exist *in vivo* between the wt U3 and wt U5 LTR termini in retroviruses as well as purified retrovirus preintegration complexes that promoted full-site integration *in vitro*.

INTRODUCTION

The retrovirus integrase (IN) is responsible for integration of the viral DNA into the host genome (Brown, 1998; Goff, 1992; Kulkosky and Skalka, 1994). Before integration, the viral RNA is reverse transcribed into a linear double-stranded DNA copy containing long terminal repeats (LTRs) at its termini. The viral DNA and IN assemble into highly ordered nucleoprotein complexes termed the preintegration complex (PIC) (Brown *et al.,* 1987; Farnet *et al.,* 1991; Lee and Coffin, 1991). The subunit composition of IN within the PIC is unknown, but it is believed to be a higher order multimer of IN (Ellison *et al.,* 1995; Jones *et al.,*, 1992) acting as a protein bridge holding the two viral LTR termini together (Miller *et al.,* 1997; Wei *et al.,* 1997, 1998). Within the PIC, IN cleaves a dinucleotide from each blunt-ended LTR terminus to expose a highly conserved 3′ recessed CA-OH moiety (Fujiwara and Mizuuchi, 1988). The full-site integration reaction (concerted insertion of the two LTR termini into the host chromosome) is then carried out by IN via a transesterification reaction (Mizuuchi, 1992), resulting in the proviral intermediate.

IN must bind to the viral LTR termini for strand transfer to occur; however, IN also binds DNA nonspecifically (Vink and Plasterk, 1993). The mechanism for specific recognition of viral LTR termini by IN to perform full-site strand transfer is unknown. Among all retroviruses, there is a conservation of a CA dinucleotide located two bp from the blunt-ended LTR termini. The presence of this CA dinucleotide is not sufficient to promote efficient strand transfer activity; therefore, additional internal LTR sequences are required. Experiments with oligonucleotides to measure half-site strand transfer activities (insertion of a single LTR terminus into target DNA) suggest that the terminal 5–7 bp of the LTR inverted repeats are essential for IN recognition and strand transfer (Balakrishnan and Jonsson, 1997; Brown, 1998; Scottoline *et al.,* 1997; Yoshinaga and Fujiwara, 1995).

Within the avian retrovirus DNA, the terminal 15 bp of the U3 and U5 LTR termini form imperfect inverted repeats. IN exhibits a preference for the U3 LTR over the U5 LTR for removal of the dinucleotide and for half-site and full-site strand transfer *in vitro* (Vora and Grandgenett, 1995; Vora *et al.,* 1994, 1997). Mutation analyses have shown that the fifth nucleotide from the blunt-ended LTR terminus appears to be responsible for the preference of U3 over U5 LTR termini by IN (Vora *et al.,* 1997).

Mutation analysis of Moloney murine leukemia virus

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TABLE 1

Summary of Gain- and Loss-of-Function LTR Donor Substrates[a]

Name	Sequence[b] U3 LTR	Sequence[b] U5 LTR	Designation[c]
wt U3-U5	5′-TATGTAGTCT	AGGCTTCA$_{OH}$	U3-U5
	$_{HO}$ACATCAGA	TCCGAAGTAT-5′	
U3P6-T/A	$_{HO}$ACA**A**CAGA	AGGCTTCA$_{OH}$	U3/G-U5
U3P5-A/T	$_{HO}$AC**T**TCAGA	AGGCTTCA$_{OH}$	U3/L-U5
U3P5-A/T:U5P5, 6-TT/AA	$_{HO}$AC**T**TCAGA	AGGC**AA**CA$_{OH}$	U3/L-U5/G
U3P6-T/A:U3P6-T/A	$_{HO}$ACA**A**CAGA	AGAC**A**ACA$_{OH}$	U3/G-U3/G
*Hin*fI-U3/L	$_{HO}$AC**T**TCAGA — *Hin*fI		U3/L
*Hin*fI-U5/G	*Hin*fI —	AGGC**AA**CA$_{OH}$	U5/G
U3Δ6,P8A/G-U5[d]	$_{HO}$ACA**CGGA**A	AGGCTTCA$_{OH}$	U3Δ6,P8A/G-U5
U3ΔP10-U5[d]	$_{HO}$ACATCAGA	AGGCTTCA$_{OH}$	U3ΔP10-U5

[a] Donor substrates used for *in vitro* strand transfer assay.

[b] Mutant LTR donors have only the catalytic LTR strand indicated. Sequence changes from wt are indicated in bold. Numbers indicate distance from the blunt-ended termini.

[c] Names containing two LTR references indicate the presence of two LTR termini on the donor. The G and L indicate whether the LTR mutation resulted in a gain- or loss-of-function, respectively.

[d] Sequences of mutant U3 LTR termini shown in Fig. 4.

(M-MLV) DNA *in vivo* shows that a 7-bp deletion of one LTR (U3 terminal nucleotides 5–11) blocks the 3′ OH processing of both blunt-ended LTR (Murphy and Goff, 1992). This result suggests that the two LTR termini are capable of interacting *in trans* across a protein bridge in the M-MLV PIC that is likely directed by IN (Wei *et al.,* 1998). The specificity for these interactions requires at least the terminal ~12 bp of the LTR, to varying degrees, for the full-site integration *in vivo* (Masuda *et al.,* 1998; Murphy and Goff, 1992; Reicin *et al.,* 1995). *In vivo*, HIV-1 IN appears to recognize the U3 and U5 LTR termini independently, suggesting that the initial binding of IN at each LTR terminus may occur before producing paired LTR termini (Masuda *et al.,* 1998).

The exact mechanisms involved in the assembly of the PIC, the recognition of the U3 and U5 LTR termini by IN, and the communication mechanisms that exist between the LTR termini coupled by IN remain unknown. In this report, we studied the interactions that occur between two LTR termini bound by IN for full-site strand transfer activity. We used avian myeloblastosis virus (AMV) IN and retrovirus-like DNA substrates having either wild-type (wt) or mutant LTR termini previously identified to cause either a gain-of-function ("G") or a loss-of-function ("L") for strand transfer activities (Vora *et al.,* 1997) (Table 1). The L mutations are classified by their reduction of strand transfer activity and further classified by their ability to either permit or block assembly of nucleoprotein complexes capable of full-site strand transfer activity. We used restriction digestion and agarose gel electrophoresis to show that a G LTR terminus increased the inclusion of a partially defective L LTR terminus into nucleoprotein complexes that promoted full-site strand transfer. The observed full-site integration activity of these assembled complexes appeared to be controlled by both of the individual IN–LTR complexes *in trans*. Significantly defective LTR ends effectively blocked the inclusion of wt LTR ends into nucleoprotein complexes capable of full-site strand transfer.

RESULTS

Strategy for studying the interactions that occur between LTR termini bound by IN for full-site integration

We wanted to investigate the functional roles that LTR termini have in nucleoprotein complexes that are capable of full-site strand transfer *in vitro*. Our reconstitution experiments included purified AMV IN and LTR donor substrates that contained mutations in the inverted repeats sequences. Mutations introduced into the fifth and sixth nucleotides in the inverted repeats of the wt U3 and wt U5 LTR termini conferred either a G or L for full-site and half-site strand transfer activities (Vora *et al.,* 1997). G LTR termini are characterized as having significantly higher catalytic activity than the wt and the L LTR termini. Table 1 contains the wt and a list of mutated LTR donor substrates (480 bp in length) along with their essential terminal LTR sequences. These LTR mutations allowed us to gain insights into the interactions that occur between paired LTR termini bound by IN that promote the full-site integration reaction (Fig. 1).

Besides the well-established effect that LTR sequences have on the observed activities associated with IN, the protein concentration in the reconstitution experiments also plays an important role. Standard strand transfer reactions were performed with a G donor (U3/G-U5) and the wt U3-U5 donor with different concentrations of IN (Fig. 2). With a

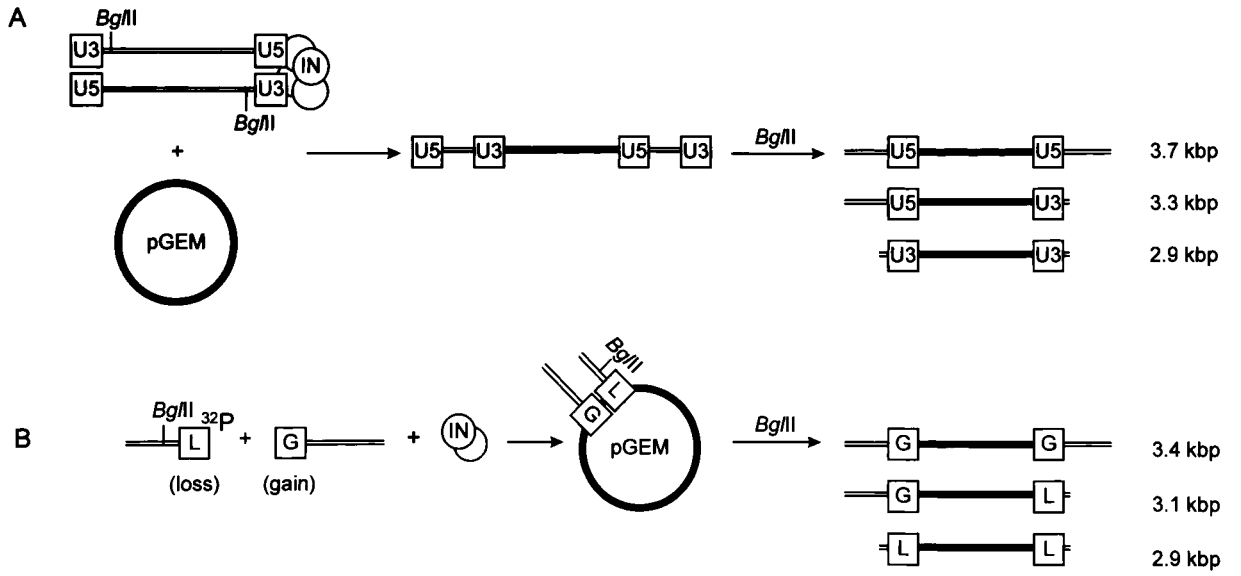

FIG. 1. Schematic of the full-site strand transfer assay. (A) The reaction pathway using 480-bp donors possessing two LTR termini (double-ended donor). The U3 and U5 LTR termini are indicated in the boxes with a *Bgl*II restriction site located near the U3 LTR (left). Donors are complexed with IN and the reaction is initiated by the addition of pGEM as target (2867 bp). The linear full-site product (3.8 kb) produced (middle) is digested with *Bgl*II, yielding three DNA fragments (right) dependent on the orientation of the LTR termini inserted by IN into pGEM. For example, the largest U5-U5 product (3.7 kbp) is the result of *Bgl*II removal of the U3 LTR ends. The *Bgl*II site is only 42 bp from the U3 end. (B) Reaction pathway using donors with a single LTR terminus (single-ended donor). The single-ended LTR donors have either G or L LTR end mutations. The single-ended LTR donors were produced by *Hin*fI digestion of the double-ended LTR donor. To test the effect of one LTR on another *in trans,* only one single-ended LTR donor is labeled with ^{32}P for detection. The linear full-site bimolecular products (not shown) can be digested with *Bgl*II to identify the orientation of G and L insertions into the target. In the example shown, only full-site products with a ^{32}P-labeled L LTR end can be detected by autoradiography.

single nucleotide change (sixth position, T to A) in the U3 LTR terminus of U3/G-U5 (Table 1), the incorporation of this donor into the target was increased approximately threefold over the U3-U5 donor at 50 nM IN. Higher concentrations of IN (~60 nM or more) under standard assay conditions result in inhibition of full-site strand transfer activity to a faster degree than half-site activity (Fig. 2) (Vora *et al.,* 1995, 1997) (data not shown).

LTR termini bound by IN influence each other's ability to participate in complexes capable of full-site integration activity

Concerted integration involves the simultaneous insertion of two LTR termini paired by IN into the target. Previous results suggested that two LTR termini coupled by IN affect each other's activities for full-site strand transfer (Aiyar *et al.,* 1996; Vora *et al.,* 1994, 1997). We wanted to examine how individual IN–LTR complexes affected each other's ability to assemble into nucleoprotein complexes capable of full-site integration. An LTR donor with identical LTR termini on both ends (U3/G-U3/G) (Fig. 3, set 1) served as the control substrate, and a donor with L and G LTR termini (U3/L-U5/G) (Fig. 3, set 2) served as the test substrate (Table 1, top). The major faster migrating DNA in the undigested samples is the linear full-site product (3.8 kb), whereas the slower migrating DNA is the circular half-site product (Fig. 3, − lanes in sets 1 and 2). At 50 nM IN, the total amount of donor incorporated into the full-site products for U3/G-U3/G and U3/L-U5/G was estimated to be 26% and 12%, respectively (data not shown, see Fig. 2 as an example).

The full-site donor-target products were subjected to *Bgl*II restriction analysis to determine the frequency of the paired LTR termini (Fig. 1A). As anticipated with the U3/G-U3/G donor (Fig. 3, + lane in set 1), the three digested full-site fragments (left, 3.7, 3.3, and 2.9 kb in length) have near ratios of 1:2:1 (Vora *et al.,* 1997). These data suggest that all of the U3/G LTR termini bound by IN have an equal probability to participate in full-site strand transfer. Thus the distribution of donor ends of the test donor (see below) into the target would be a direct reflection of two interacting LTR termini for full-site integration. In the same + lane (Fig. 3, set 1), the two *Bgl*II digested half-site products of the U3/G-U3/G donor were also equivalent in quantity to each other.

We wanted to determine whether a G LTR terminus (U5/G) was able to increase the inclusion of an L LTR end (U3/L) into nucleoprotein complexes capable of full-site strand transfer. In the test donor (U3/L-U5G) (Fig. 3, set 2), *Bgl*II digestion demonstrated a significantly different digestion pattern (see right side of photograph for *Bgl*II fragments of set 2) derived from its linear full-site 3.8-kb product than that observed with the U3/G-U3/G donor (set 1). From the largest to the smallest in size, the ratios of the full-site products were 1:0.8:0.03, respec-

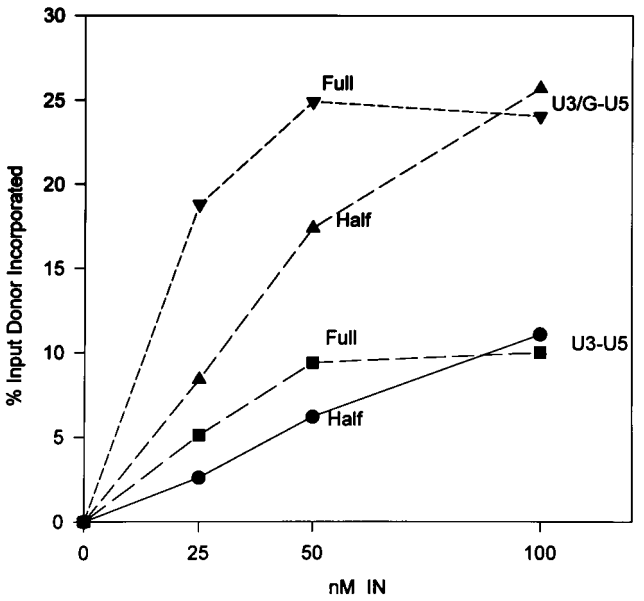

FIG. 2. Strand transfer efficiencies of U3/G-U5 and U3-U5 donors at varying IN concentrations. Standard reaction conditions were used with LTR donors having wt U3 and U5 LTR termini (U3-U5) or a wt U5 LTR terminus tethered to a G U3 LTR terminus (U3/G-U5). After preincubation of IN with the ^{32}P-labeled donors on ice, reactions were initiated by the addition of pGEM and incubation at 37°C for 10 min. Aliquots of each reaction were subjected to electrophoresis on 1% agarose gels to separate the half-site and full-site products. The dried gels were subjected to PhosphorImager analysis. Percent incorporation of the donors was determined by calculating the number of pixels in each donor/target product divided by the total pixels in each lane. Approximately 1% of the input donor is used to produce donor/donor products (Vora and Grandgenett, 1995).

tively (Fig. 3, + lane in set 2) in contrast to the ratio (1:2:1) observed in the set 1 control experiment. These data suggest that several different types of interactions are occurring *in trans* between the U3/L and U5/G termini. First, the amount of the homologous U5/G-U5/G full-site product (3.7 kb) was significantly higher that the homologous U3/L-U3/L full-site product (2.9 kb). Second and more importantly, IN bound to the more active G U5/G terminus was able to interact *in trans* with IN bound to the less active L U3/L terminus to produce a significant quantity of the 3.3-kb U3/U5 fragment (3.3 kb). Last, the observed full-site activity of the nucleoprotein complexes that produced the 3.3-kb U3/U5 fragment appears to be controlled by both the more active IN–U5/G complex and the less active IN–U3/L complex *in trans*. As expected, the U5/G half-site reaction was significantly greater than the less active U3/L half-site reaction in the same reaction mixture (Fig. 3, set 2, + lane).

To determine that IN was not limiting in any of the reactions shown in Fig. 3, the above experiments were repeated at 25, 50, 100, and 200 nM IN (data not shown). As stated previously, the full-site reactions were inhibited at these higher concentrations of IN, but the ratios of the *Bgl*II digestion fragments of both LTR donors shown in Fig. 3 did not change significantly.

In summary, IN bound to a more active LTR terminus increases the inclusion of IN bound to a less active LTR terminus in nucleoprotein complexes capable of full-site strand transfer. These interactions appear to occur *in trans* to promote assembly of competent nucleoprotein complexes. The observed full-site integration activities of these nucleoprotein complexes (G/G, G/L, and L/L reactions) (see right side of Fig. 3, set 2) appear to be the result of activities associated with each participating IN–LTR complex. Importantly, similar regulatory controls appear to be occurring between wt U3 and wt U5 LTR termini to produce their U5/U5, U3/U5, and U3/U3 products (Fig. 4, lanes 1 and 2).

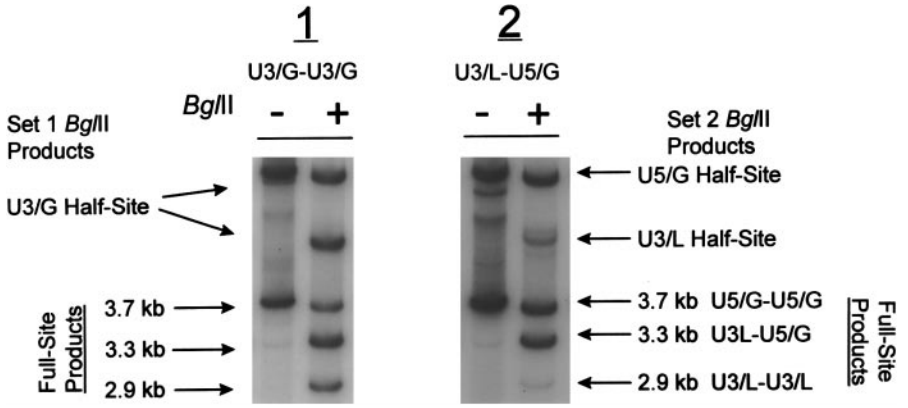

FIG. 3. LTR termini interact with each other to influence assembly of nucleoprotein complexes capable of full-site strand transfer activity. The ^{32}P-labeled LTR donors were bound to IN and inserted into pGEM under standard reaction conditions. Set 1 contains the control U3/G-U3/G LTR donor. Set 2 contains the test U3/L-U5/G LTR donor. After preparation, half the samples for each set were digested with *Bgl*II restriction enzyme as indicated by the + lanes, whereas the other half were untreated (− lanes). Products were separated by electrophoresis on 1.5% agarose gels and detected by autoradiography. In both sets, the half-site (top band) and the 3.8-kb full-site (bottom band) products are in the undigested lanes. *Bgl*II digested half-site and full-site fragments obtained with the U3/G-U3/G LTR donor (set 1) and U3/L-U5/G LTR donor (set 2) are indicated on the left and right sides, respectively. For both donors, the digested products are 3.7, 3.3, and 2.9 kb in length.

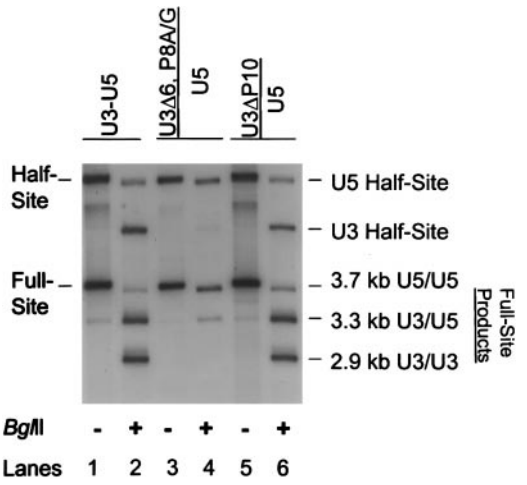

FIG. 4. A significantly defective L LTR terminus effectively blocks inclusion of a wt LTR terminus into nucleoprotein complexes capable of full-site strand transfer. The wt U3-U5, U3Δ6, P8A/G-U5, and U3ΔP10-U5 donors (top) were subjected to strand transfer analysis with AMV IN under standard assay conditions. The samples were not (−) (odd number lanes) or were (+) (even number lanes) digested with *Bgl*II and analyzed on 1.5% agarose gels. The half-site and full-site products are indicated on the left side, whereas the *Bgl*II fragments are indicated on the right side of the photograph. The 3.7-, 3.3-, and 2.9-kb fragments are derived from each donor set, but only the wt donor/target products were marked on the right. The mutant U3/U5 and U3/U3 LTR products were not marked on the right but are inferred from each mutant LTR donor as indicated at the top.

Requirement of two good LTR termini to mediate full-site integration

As shown in Fig. 3 (set 2), the test donor (U3/L-U5/G) was efficient in making full-site U3/L-U5/G products (3.3 kb) even though the U3/L terminus had low homologous U3/L-U3/L full-site activity (2.9-kb product). The simplest explanation is that IN is bound to U3/L in such a matter that allows it to interact fully with either G or L LTR termini.

In the following experiment, we directly addressed whether one LTR terminus that is nearly defective for half-site integration can effectively interact with a wt U5 LTR terminus. The wt U3 LTR sequences were significantly modified, whereas the other end of the 480-bp donor had wt U5 sequences (Table 1, bottom). The sixth nucleotide (T) was deleted, and the eighth nucleotide (A) was switched to G, which produced the U3Δ6,P8A/G-U5 donor (Fig. 4). The control reaction of a wt U3-U5 donor produced the usual *Bgl*II restriction pattern for U5/U5 (3.7 kb), U3/U5 (3.3 kb), and U3/U3 (2.9 kb) products with near molar ratios of 1:3.6:5.4, respectively (Fig. 4, lane 2) (Vora *et al.,* 1994, 1997). With the test donor U3Δ6,P8A/G-U5, the mutant U3 LTR half-site and homologous U3/U3 full-site products (2.9 kb) were just apparent (Fig. 4, lane 4) and significantly below the wt U3 reactions observed with the U3-U5 donor (lane 2). Although not entirely, the defective U3 LTR effectively blocked the incorporation of the wt U5 LTR terminus into nucleoprotein complexes that produced the U3/U5 product (3.3 kb) shown in lane 4. The wt U5 LTR terminus on the mutant U3 donor (Fig. 4, lane 4) was fully functional for its half-site and homologous full-site (3.7 kb product) reactions, suggesting that IN preferentially interacts with the wt U5 rather than the defective U3Δ6,P8A/G end. Similar blocking data were obtained with another 480-bp LTR donor that had a significantly defective U3 LTR terminus (nucleotides 7–10 were deleted) and a wt U3 terminus (data not shown). A single base pair deletion at the 10th nucleotide in U3 slightly decreased its half-site activity but had little effect on its U3/U5 or U3/U3 full-site products (Fig. 4, lane 6) (Vora *et al.,*1997).

In summary, IN does not have the ability to effectively incorporate significantly defective U3 LTR ends into nucleoprotein complexes containing wt LTR ends that are capable of promoting full-site catalysis (Fig. 4, lane 4). With this experimental approach, it is not possible to define the defect that exists on the mutant U3 ends (binding to it by IN or inactive sequences, or both). These data are consistent with results that show two functional LTR termini are required for M-MLV IN to promote 3′ OH processing of both blunt-ended LTR termini *in vivo* (Murphy and Goff, 1992) or IN-promoted footprint enhancements and protection of both LTRs in isolated M-MLV PIC (Wei *et al.,* 1998).

Conditions for assembling and maintaining stable IN–DNA complexes capable of full-site integration activity

We determined that AMV IN requires two functional LTR termini (G or L) to efficiently promote full-site strand transfer (Fig. 3). A wt LTR terminus is not capable of pairing with a significantly defective L LTR terminus to promote the full-site integration reaction (Fig. 4). We wanted to investigate whether IN is capable of forming nucleoprotein complexes with a G LTR end significantly better than an L LTR end in the presence of increasing concentrations of nonspecific DNA. We used U3/G-U3/G (Fig. 3) and U3/L-U5 (Table 1) as G and L LTR donors, respectively. The competitor DNA (*Hpa*II fragment derived from pGEM) was equivalent in size and had 5′ 2-bp overhangs similar to the LTR donors, and the termini lacked LTR sequence homology.

Under standard assay conditions with 50 nM IN, the amount of donor incorporated into full-site products were 34% and 8% for the U3/G-U3/G and U3/L-U5 donors, respectively (data not shown). This condition was also defined as 100% efficiency (when no competitor was present) (Fig. 5). The 5′ end-labeled U3/G-U3/G or U3/L-U5 donors were present with 0-, 1-, 5-, or 10-fold molar excess of competitor DNA before preincubation with 50 nM IN on ice. Incorporation of the U3/G-U3/G donor was reduced to 32% when 10-fold molar excess competitor was present, whereas the U3/L-U5 donor was reduced to

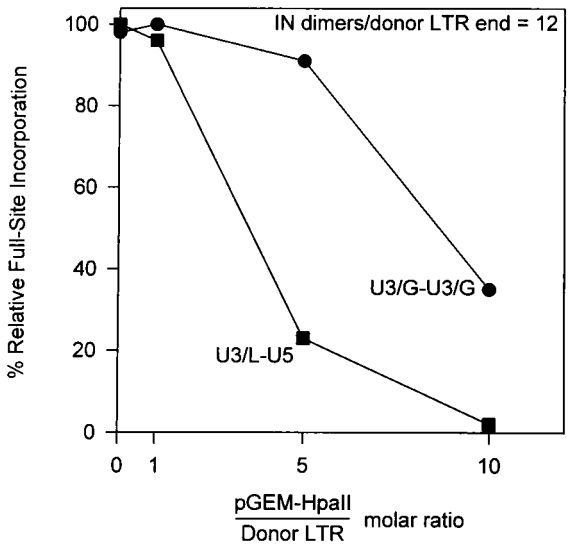

FIG. 5. Effect of nonspecific DNA competition on G and L LTR donor directed full-site strand transfer. Double-ended LTR donors labeled with ^{32}P were used in a standard strand transfer assay. Full-site strand transfer efficiencies were calculated by PhosphorImager analysis, and the maximal efficiency for each donor was set at 100%. Strand transfer mixtures contained 50 nM IN and 15 ng of either ^{32}P-labeled U3/G-U3/G or U3/L-U5 LTR donors. IN donor complexes were formed on ice in the presence of varying amounts of the *Hpa*II digested pGEM fragment. After incubation for 10 min at 37°C with pGEM, samples were removed and subjected to electrophoresis on 1% agarose gels. The change in relative full-site strand transfer activities were plotted against the molar ratio of pGEM-*Hpa*II fragment to LTR donor (15 ng of pGEM-*Hpa*II fragment and 15 ng of LTR donor equals a 1:1 molar ratio, respectively).

2% at this same competitor level. These data suggest that IN has an apparent higher affinity for G LTR termini over L LTR termini in the presence of nonspecific DNA as competitor.

The dissociation of IN from the U3/L-U5 donor ends may have been significantly faster than that from U3/G-U3/G in the above competition experiment, resulting in the differential decreased for full-site catalysis (Fig. 5). We tested the stability of two different IN–LTR donor complexes on ice in the absence of competitor DNA (or at a molar ratio of 1 with the *Hpa*II fragment) at 25 nM and 50 nM IN. The half-life of the U3/L-U5 and U3-U5 donors for promoting both of their respective half-site and full-site reactions was ~2 h (data not shown). These data suggest that the apparent dissociation rate of IN from the each LTR terminus was sufficiently slow, therefore, no difference was observed in the stability of either IN–LTR complexes.

Modulation of full-site strand transfer activity by order of addition of LTR substrates and IN

We wanted to investigate how the order of addition of naked LTR donors affects full-site strand transfer of IN–DNA complexes already subjected to preincubation for 10 min. At 50 nM IN, the maximum amount of IN–LTR complexes assembled for full-site strand transfer on ice is rapid (~1 min) (Vora and Grandgenett, 1995) (data not shown). Single-ended LTR donors were used instead of double-ended LTR donors to simplify the data obtained after *Bgl*II digestion of the donor-target products. The U3/L-U5/G donor (Fig. 3) was 5′ end-labeled and digested with *Hin*fI *to* separate the two fragments containing the LTR ends (Fig. 1B). The U5/G and the partially defective U3/L fragment sizes were 259 and 184 bp, respectively (Figs. 6A and 6B) (Table 1). The internal *Hin*fI site of the donor lacks LTR sequence homologies, but IN probably binds to the *Hin*fI end in a nonspecific fashion (Knaus *et al.,* 1984).

Control strand transfer reactions were first performed with the isolated single-ended LTR donors themselves (Fig. 6A, sets 2 and 3). The concentration of each donor fragment (one end is nonspecific) was varied to maintain the 12:1 IN-to-donor end ratio with 50 nM IN, respectively. The amounts of input donor incorporated into full-site products (fastest migrating DNA) using U5/G and U3/L LTR donors were 10% and ~1%, respectively (see the − *Bgl*II lanes in sets 2 and 3 of Fig. 6A). The U5/G full-site product lacks a *Bgl*II site, whereas the U3/L full-site product contains a *Bgl*II restriction site, and therefore, the latter was modified by restriction digestion (+ *Bgl*II lanes). A standard double-ended wt U3-U5 donor reaction was performed at the same time and is shown in set 1 (Fig. 6A) for comparison. The above results show that single-ended LTR donors are capable of promoting full-site catalysis and that their *Bgl*II digestion patterns can be used to address order of addition and *trans* interactions between different IN–LTR complexes.

After assay conditions were established (Fig. 6A, sets 2 and 3), order of addition experiments were performed using an unlabeled single-ended U5/G fragment and a labeled U3/L fragment (Fig. 6B). Unlabeled U5/G DNA was used to allow easier identification of undigested products having the same size and of *Bgl*II digested donor-target full-site products. In the undigested products (Fig. 6B − in all sets), there are two closely migrating DNA fragments (~3.3 kb) at different proportions depending on the order of addition. *Bgl*II digestion of the DNA products (Fig. 6B, + lanes in all sets) clearly defined the U5/G-target-U3/L product (3.1 kb). Preincubation of labeled U3/L and IN with either unlabeled U5/G present before (Fig. 6B, set 1) or after complex formation (Fig. 6B, set 2) resulted in an approximately threefold increase in the 3.1-kb full-site product (G/L) compared with the 2.9-kb full-site product (L/L). Preincubation of unlabeled U5/G with IN followed by the addition of labeled U3/L also resulted in enhanced production of the 3.1-kb full-site product, although to a lesser degree (Fig. 6B, set 4). These data suggest that not all of IN is complexed to the single-ended LTR donor in a stable fashion. Some of IN is likely bound internally or at the

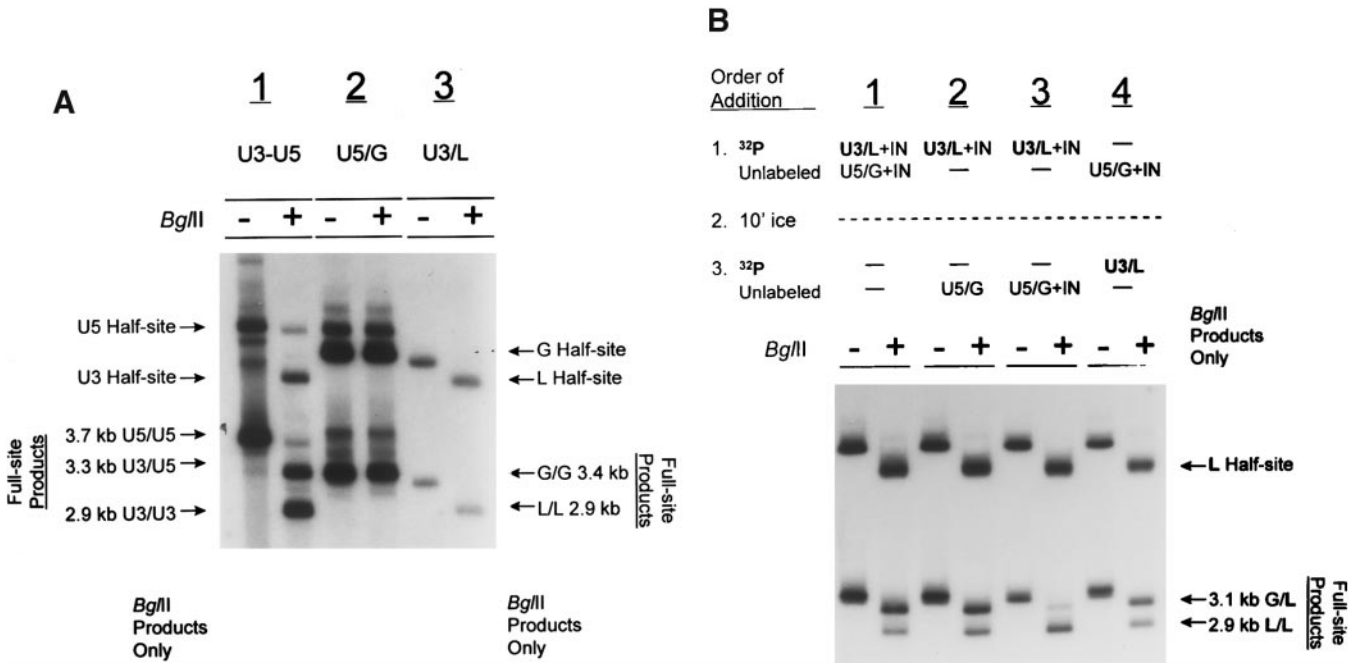

FIG. 6. IN–LTR complexes capable of full-site strand transfer activity competed with naked LTR DNA. (A) Control reactions containing ^{32}P-labeled double-ended U3-U5 LTR donor, single-ended U5/G LTR donor, or single-ended U3/L LTR donor (sets 1–3, respectively). Lanes with *Bgl*II digested samples are indicated by a +, and undigested samples are indicated with a −. The left margin denotes the U3-U5 LTR donor products (set 1), and the right margin indicates the single-ended LTR donor products (sets 2 and 3) after *Bgl*II digestion only. (B) IN–LTR donor complexes capable of full-site strand transfer competed with naked LTR DNA. The ^{32}P-labeled (bold lettering) and unlabeled competitor LTR donors were added at various times in the preincubation step before strand transfer. After mixing of preincubated samples, reactions were initiated by the addition of pGEM and incubation at 37°C for 10 min. Set 1, ^{32}P-labeled U3/L and unlabeled U5/G preincubated together with IN for 10 min before adding target. Set 2, ^{32}P-labeled U3/L preincubated with IN for 10 min followed by the addition of unlabeled U5/G just before initiation of strand transfer. Set 3, ^{32}P-labeled U3/L preincubated with IN and mixed with unlabeled U5/G preincubated separately with IN on ice just before strand transfer. Set 4, unlabeled U5/G preincubated with IN followed by the addition of ^{32}P-labeled U3/L just before initiation of strand transfer.

*Hin*fI end, particularly because the normal IN-donor end ratios (12:1, respectively) were used in this experiment.

Significantly different results were obtained when two independently formed IN–LTR complexes were mixed together (Fig. 6B, set 3). Addition of preformed IN–U5/G complexes to preformed IN–U3/L complexes followed by the addition of target for strand transfer resulted in reduced formation of the 3.1-kb DNA product (Fig. 6B, + *Bgl*II lane in set 3). Allowing the two independently formed IN–LTR complexes to preincubate together on ice for up to 20 min before strand transfer did not enhance the production of the 3.1-kb product (data not shown). As previously stated, these data suggest that once IN–LTR complexes capable of full-site strand transfer are formed on ice, they are quite stable and are not readily exchangeable with each other.

In summary, IN is capable of binding to either partially defective L (U3/L) or G (U5/G) LTR termini in a stable fashion, which allows inclusion of these termini into nucleoprotein complexes capable of full-site strand transfer (Figs. 3 and 6B).

DISCUSSION

We have shown that a G LTR terminus bound by IN increases the inclusion of a partially defective L LTR terminus into nucleoprotein complexes capable of full-site integration *in trans*. The observed full-site strand transfer activity of these nucleoprotein complexes appears to be a combination of the two independently paired IN–LTR complexes. Paired IN–LTR complexes containing either G or L LTR termini are stable on ice, similar to single IN–LTR, complexes that promote only the half-site strand transfer reaction. Significantly defective LTR termini were not capable of interacting with wt LTR termini to promote efficient pairing and full-site strand transfer.

Juxtaposition of the retrovirus LTR termini by IN is required for promoting the full-site integration reaction. The protein–DNA and protein–protein interactions required for the proper alignment of the LTR termini by IN in the PIC or in the reconstituted nucleoprotein complexes studied in this report are not fully understood. We have demonstrated that an LTR terminus bound by IN significantly increases the inclusion of and the catalytic activity of another IN–LTR complex *in trans* within nucleoprotein complexes that promote full-site integration (Figs. 3, 4, and 6). These data suggest that the protein–protein interactions that occur between paired AMV IN–LTR complexes may be similar to the interactions that appear to occur between paired LTR termini within the

M-MLV PIC *in vivo* (Murphy and Goff, 1992) or with the purified M-MLV PIC *in vitro* (Wei *et al.,* 1998). Recent footprint analyses on M-MLV viral DNA ends in purified PIC suggest a specific association by IN at the viral LTR termini and for several hundred nucleotides internal to the termini, whereas a cellular protein (barrier-to-autointegration factor) has an indirect role in the footprints (Wei *et al.,* 1998). Our reconstitution experiments using purified components suggest that IN alone may be sufficient for efficient juxtaposition of the two LTR termini. IN is capable of looping DNA by either intramolecular or intermolecular mechanisms (Grandgenett *et al.,* 1993). The roles that IN and possibly cellular proteins have in the juxtaposition of the LTR termini for full-site activity and in holding together the two LTRs in the PIC (Li *et al.,* 1998; Miller *et al.,* 1997; Wei *et al.,* 1997, 1998) need further study.

Recombinant HIV-1 IN forms stable complexes with oligonucleotides containing 3′ OH recessed LTR termini but not with blunt-ended LTR termini (Ellison and Brown, 1994). The nucleoprotein complexes assembled by AMV IN with wt, G and L LTR donor substrates containing 3′ OH recessed LTR termini are also stable. Formation of AMV IN-single-ended LTR complexes may be a requirement for assembly of paired IN–LTR complexes *in vitro* (Figs. 2 and 6), with U3 having a leading role over U5 (Vora *et al.,* 1997). Once the termini are paired together into nucleoprotein complexes capable of full-site strand transfer, both LTR termini appear to contribute to the observed activities (Figs. 3 and 6). Similar regulatory properties appear to occur between two LTR termini bound by IN *in vivo* (Murphy and Goff, 1992) and in the purified M-MLV PIC (Wei *et al.,* 1998).

The short inverted repeats at the LTR termini play critical roles for 3′ OH processing and strand transfer activities (Brown, 1998; Kulkosky and Skalka, 1994). The fifth and sixth nucleotides of the avian LTR inverted repeats are also critical for recognition and subsequent full-site strand transfer (Fig. 3) (Table 1) (Vora *et al.,* 1997). The apparent association of IN is higher with G LTR mutants over partially defective L LTR mutants in the presence of nonspecific DNA (Fig. 5) or wt U3 over U5 LTR termini (Fig. 4) (Vora *et al.,* 1997). This specificity may involve both a direct readout of the DNA sequence accessible in the grooves of the helix by specific residues of IN and an indirect readout mechanism contributed from the effects of nucleotide sequence on the physical properties of the DNA site (Record *et al.,* 1991). The increase or decrease in half-site and full-site strand transfer activities of AMV IN with wt U3 and U5 termini involve the presence and the location of T and A nucleotides in the fifth and sixth positions of the invert repeats (Table 1). Modifying these two nucleotides to CC on the catalytic strand of either U3 or U5 produces significantly defective termini for half-site or full-site strand transfer activities (data not shown). How IN distinguishes T•A from A•T base pairs for affecting its association with LTR termini (Fig. 5) or for strand transfer activity (Figs. 3, 4, and 6) by one or both of the above protein–DNA recognition mechanisms needs further investigation.

The *in vivo* effects of the mutations introduced into the fifth and sixth nucleotides of the U3 and U5 LTR inverted repeats used in this study (Table 1) have not been fully investigated. Modifications of nucleotides at these positions in several retroviruses affect virus replication, usually resulting in slower growth phenotypes relative to wt virus (Brown, 1998; Goff, 1992). Substitution of TT with AA in U5 at these positions (Table 1) in an avian retrovirus was not lethal but decreased the virus replication rate (Corbrinik *et al.,* 1991; Miller *et al.,* 1997). It is likely that any alteration at these two positions, in either a positive or a negative fashion for *in vitro* activities, will affect avian retrovirus replication in a negative fashion relative to wt virus replication.

MATERIALS AND METHODS

Purification of AMV IN

AMV IN was purified to apparent homogeneity as previously described (Grandgenett *et al.,* 1978; Knaus *et al.,* 1984). The protein concentration of IN preparations was calculated by measuring absorbance at 280 nm where 1 OD_{280} corresponds to a concentration of 1.87 mg/ml (McCord *et al.,* 1998).

DNA donor construction

The wt and mutant LTR donors were produced using 60-bp oligonucleotides containing LTR termini at their ends and an internal *Hin*dIII site (Vora *et al.,* 1997). The oligonucleotides were cloned into the *Nde*I site of pUC19 that had its *Hin*dIII site removed. A *supF* gene (420 bp) was inserted into the *Hin*dIII site between the U3 and U5 LTR ends that is used for genetic selection of donor-target recombinants (Vora *et al.,* 1994). After plasmid purification, the DNAs were digested with *Nde*I to release the 480-bp donor fragments (Fig. 1A). The donor fragments were purified by agarose gel electrophoresis and Wizard PCR DNA purification kits (Promega). All of the donor molecules contained a *Bgl*II restriction site near the U3 LTR terminus that was used for restriction analysis of strand transfer products. To generate the variable length single-ended LTR donors, double-ended donors were digested with restriction enzymes. The shorter LTR donors were also purified by agarose gel electrophoresis. The target DNA was supercoiled pGEM3 that lacks a *Bgl*II restriction site.

Labeling of DNA

The double-ended LTR donors were 5′ end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Vora *et al.,* 1994). The single-ended LTR donors were derived

from the labeled double-ended LTR donors resulting in equivalent labeled LTR fragments. The specific activities were ~20,000 cpm/ng of DNA. Labeled donors were used to track the integration reactions by autoradiography.

Strand transfer conditions

The standard reaction conditions for full-site strand transfer were previously described (Vora and Grandgenett, 1995). A reaction mixture (20 μl) contained AMV IN at 50 nM and 15 ng of donor DNA. The IN dimer-to-donor end ratio was 12:1, respectively. The mixture was preincubated on ice for 10 min to assemble nucleoprotein complexes. To initiate strand transfer, 100 ng of pGEM target (1:1 donor-to-target molar ratio, respectively) was added to the mixture followed by immediate incubation at 37°C for 10 min. The reactions were stopped, and the DNA products were analyzed by electrophoresis on 1% agarose gels (Vora *et al.,* 1995). The DNA products were also purified, digested with *Bgl*II, and further analyzed by 1.5% agarose gel electrophoresis. A Molecular Dynamics STORM PhosphorImager was used to determine the quantities of each product.

In several experiments, nonspecific and LTR-specific DNA competition experiments were performed with several LTR donors as substrates for full-site strand transfer. Varying concentrations of nonspecific DNA was incubated together with either G or L DNA donors before the addition of IN and subsequent strand transfer (Fig. 5). The nonspecific DNA mimic the LTR donor DNA in size and had a 5′ 2-bp overhang but lack LTR sequence homology. In other experiments, single-ended LTR donors containing either G or L LTR ends were preincubated with IN to form nucleoprotein complexes that were challenged with either naked LTR donors or LTR donors bound by IN subsequent to strand transfer (Fig. 6).

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REFERENCES

Aiyar, N., Hindmarsh, P., Skalka, A. M., and Leis, J. (1996). Concerted integration of linear retroviral DNA by the avian sarcoma virus integrase *in vitro*: Dependence on both long terminal repeat termini. *J. Virol.* **70,** 3571–3580.

Balakrishnan, M., and Jonsson, C. B. (1997). Functional identification of nucleotides conferring substrate specificity of retroviral integrase reactions. *J. Virol.* **71,** 1025–1035.

Brown, P. 0. (1998). Integration *In* "Retroviruses" (Coffin, J. M., Hughes, S. J., and Varmus, H. E., Eds.), pp. 161–203, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Brown, P. 0., Bowerman, B., Varmus, H. E., and Bishop, J. M. (1987). Correct integration of retroviral DNA *in vitro. Cell* **49,** 347–356.

Cobrinik, D., Aiyar, A. Ge, Z., Katzman, M., Huang, J., and Leis, J. (1991). Overlapping retrovirus U5 sequence elements are required for efficient integration and initiation of reverse transcription. *J. Virol.* **65,** 3864–3872.

Ellison, V., and Brown, P. O. (1994). A stable complex between integrase and viral DNA ends mediates human immunodeficiency virus integration *in vitro. Proc. Nati. Acad. Sci. USA* **91,** 7316–7320.

Ellison, V., Gerton, J., Vincent, K. A., and Brown, P. O. (1995). An essential interaction between distinct domains of HIV-1 integrase mediates assembly of the active multimer. *J. Biol. Chem.* **270,** 3320–3326.

Farnet, C. M., and Haseltine, W. A. (1991). Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J. Virol.* **65,** 1910–1915.

Fujiwara, T., and Mizuuchi, K. (1988). Retroviral DNA integration: structure of a integration intermediate. *Cell* **54,** 497–504.

Goff, S. P. (1992). Genetics of retroviral integration. *Annu. Rev. Genet.* **26,** 527–544.

Grandgenett, D. P., Inman, R., Vora, A. C., and Fitzgerald, M. (1993). Comparison of DNA binding and integration half-site selection by avian myeloblastosis virus integrase. *J. Virol.* **67,** 2628–2636.

Grandgenett, D. P.,Vora, A. C., and Schiff, R. (1978). A 32000-dalton nucleic acid-binding protein from avian retrovirus cores possesses DNA endonuclease activity. *Virology* **89,** 119–132.

Jones, K. S.,Coleman, J., Merkel, G., Laue, T. M., and Skalka, A. M. (1992). Retroviral integrase functions as a multimer and can turn over catalytically. *J. Biol. Chem.* **287,** 16037–16040.

Kulkosky, J., and Skalka, A. M. (1994). Molecular mechanism of retroviral DNA integration. *Pharmacol. Ther.* **61,** 185–203.

Knaus, R. J., Hippenmeyer, P. J., Misra, T. M., Grandgenett, D. P., Muller, R., and Fitch, W. M. (1984). Avian retrovirus pp32 DNA binding protein: Preferential binding to the promoter region of long terminal repeat DNA. *Biochemistry* **23,** 350–359.

Lee, Y. M. H., and Coffin, J. M. (1991). Relationship of avian retrovirus DNA synthesis to integration *in vitro. Mol. Cell. Biol.* **11,** 1419–1430.

Li, L., Farnet, C. M., Anderson, W. F., and Bushman, F. D. (1998). Modulation of activity of Moloney murine leukemia virus preintegration complexes by host factors *in vitro. J. Virol.* **72,** 2125–2131.

Masuda, T., Kuroda, M. J., and Harada, S. (1998). Specific and independent recognition of U3 and U5 *att* sites by human immunodeficiency virus type 1 integrase *in vitro. J. Virol.* **72,** 8396–8402.

McCord, M., Stahl, J., Mueser, T. C., Hyde, C. C., Vora, A. C., and Grandgenett, D. P. (1998). Purification of recombinant Rous sarcoma virus integrase possessing physical and catalytic properties similar to virion-derived integrase. *Prot. Purif. Expression* **14,** 167–177.

Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997). Human immunodeficiency virus type 1 preintegration complexes: Studies of organization and composition. *J. Virol.* **71,** 5382–5390.

Miller, J. T., Ge, Z., Morris, S., Das, K., and Leis, J. (1997). Multiple biological roles associated with the Rous sarcoma virus 5′ untranslated RNA U5-IR stem and loop. *J. Virol.* **71,** 7648–7656.

Mizuuchi, K. (1992). Polynucleotidyl transfer reactions in transpositional DNA recombination. *J. Biol. Chem.* **30,** 21273–21276.

Murphy, J. E., and Goff, S. P. (1992). A mutation at one end of Moloney murine leukemia virus DNA blocks cleavage of both ends by the viral integrase *in vivo. J. Virol.* **66,** 5092–5095.

Record, M. T., Ha, J. H., and Fisher. M. (1991). Analysis of equilibrium and kinetic measurements to determine thermodynamic origins of stability, specificity and mechanism of formation of site-specific complexes between proteins and helical DNA. *Methods Enzymol.* **208,** 291–343.

Reicin, A. S., Kalpana, G., Paik, S., Marmon, S., and Goff, S. P. (1995). Sequences in the human immunodeficiency virus type 1 U3 region required for *in vivo* and *in vitro* integration. *J. Virol.* **69,** 5904–5907.

Scottoline, B. P., Chow, S., Ellison, V., and Brown, P. O. (1997). Disruption of the terminal base pairs of retroviral DNA during integration. *Genes Dev.* **11,** 371–382.

Vink, C., and Plasterk, R. H. A. (1993). The human immunodeficiency virus integrase protein. *Trends Genet.* **9,** 433–438.

Vora, A. C., and Grandgenett, D. P. (1995). Assembly and catalytic

properties of retrovirus integrase-DNA complexes capable of efficiently performing concerted integration. *J. Virol.* **69,** 7483–7488.

Vora, A. C., McCord, M., Fitzgerald, M., Inman, R. B., and Grandgenett, D. P. (1994). Efficient concerted integration of retrovirus-like DNA in vitro by avian myeloblastosis virus integrase. *Nucleic Acids Res.* **22,** 4454–4461.

Vora, A. C., McCord, M., Goodarzi, G., Stahl, S. J., Mueser, T. C., Hyde, C. C., and Grandgenett, D. P. (1997). Avian retrovirus U3 and U5 DNA inverted repeats: role of nonsymmetrical nucleotides in promoting full-site integration by purified virion and bacterial recombinant integrases. *J. Biol. Chem.* **272,** 23938–23945.

Wei, S. Q., Mizuuchi, K., and Craigie, R. (1997). A large nucleoprotein assembly at the viral DNA mediates retroviral DNA integration. *EMBO J.* **16,** 7511–7520.

Wei, S. Q., Mizuuchi, K., and Craigie, R. (1998). Footprints on the viral DNA ends in Moloney murine leukemia virus preintegration complexes reflect a specific association with integrase. *Pro. Natl. Acad. Sci. USA* **95,** 10535–10540.

Yoshinaga, T., and Fujiwara, T. (1995). Different roles of bases within the integration signal sequence of human immunodeficiency virus type 1 in vitro. *J. Virol.* **69,** 3233–3236.